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Spatial localization of the stimulus-induced rise in cytosolic Ca^{2+} in bovine adrenal chromaffin cells

Distinct nicotinic and muscarinic patterns

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The spatial distribution of the intracellular free Ca^{2+} (Ca_i^{2+}) rise elicited by different stimuli in bovine adrenal chromaffin cells was examined in single fura-2-loaded cells. In response to the potent secretagogues nicotine and high K^+, Ca_i^{2+} was initially localized exclusively to the entire subplasmalemmal area of the cell. In response to the ineffective secretagogues, methacholine and muscarine, the rise in Ca_i^{2+} originated only in one pole of the cell and even at the peak of the response Ca^{2+} was still generally restricted to this same area of the cell. These results suggest that the triggering of exocytosis from these cells requires a specific spatial distribution of Ca_i^{2+}.

Ca^{2+}, localization; Fluorescence imaging; Fura-2; Agonist secretion; (Adrenal chromaffin cell)

1. INTRODUCTION

The bovine adrenal medullary chromaffin cell is known to possess both nicotinic [1] and muscarinic [2] cholinergic receptors. In common with other excitable cells [3], influx of external Ca^{2+} is a necessary prerequisite for the triggering of exocytosis due to depolarizing stimuli such as nicotine or high K^+ (review [4]). The role of the muscarinic receptor in secretion from this cell is less clear. Studies using cell populations have revealed that although muscarinic receptor activation results in phosphoinositide breakdown [5], inositol 1,4,5-trisphosphate ($InsP_3$) accumulation [6] and a smaller rise in $[Ca^{2+}]_i$ that is independent of extracellular Ca^{2+} [7,8], there is little or no secretory response [8,9,10,13]. It has been assumed that this failure of muscarinic stimuli to stimulate secretion reflects the fact that the modest rise in $[Ca^{2+}]_i$ is insufficient to trigger the exocytotic machinery [7,8,11]. A recent study on single cells using fluorescence imaging techniques, however, has revealed that some chromaffin cells are capable of producing a Ca^{2+} transient in response to muscarinic stimulation of comparable magnitude to that produced by nicotine [12].

In order to gain further insight into why Ca^{2+} influx, but not mobilization of internal Ca^{2+}, appears to trigger full secretion from these cells, we have used fluorescence imaging techniques to compare the spatial distribution of the Ca^{2+} signal induced by depolarizing stimuli with that induced by Ca^{2+}-mobilizing muscarinic drugs in single fura-2-loaded cells. The results show that the distribution of Ca_i^{2+}induced by depolarizing stimuli is markedly different from that induced by muscarinic agonists. Potent (depolarizing) stimuli resulted in an immediate elevation of Ca^{2+} activation throughout the entire subplasmalemmal region whereas muscarinic agonists resulted in

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Ca^{2+} being largely confined to one pole of the cell. This restricted localization of Ca^{2+} may not be capable of either triggering or maintaining the subplasmalemmal events necessary to trigger exocytosis.

2. MATERIALS AND METHODS

Chromaffin cells were isolated from bovine adrenal medullas by enzymatic digestion using either the method of Knight and Baker [14] or a modification [15] of that of Greenberg and Zinder [16]. Cells were isolated in Ca^{2+}-free Krebs-Ringer buffer consisting of 145 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 10 mM glucose, 20 mM Hepes, pH 7.4 (buffer A), and then washed in buffer A. Chromaffin cells were purified by differential plating [17]. Non-adherent chromaffin cells were then plated in 24-well trays at a density of 0.7×10^6 cells per well in culture medium (Dulbecco's modified Eagle's medium with 25 mM Hepes, 10% foetal calf serum, 8 μm fluorodeoxyuridine, 50 μg/ml gentamycin, 10 μM cytosine arabinoside, 2.5 μg/ml fungizone, 25 U/ml penicillin, 25 μg/ml streptomycin) for 2 days.

The determination of catecholamine secretion was carried out on fura-2-loaded cultured cells at 37°C as in [8,18]. Determination of cytosolic free Ca^{2+} in cell populations was performed at 37°C using fura-2 as described [19].

Imaging of intracellular Ca^{2+} in single cells was carried out as detailed in [12]. Briefly, after differential plating cells were resuspended in fresh DMEM and seeded onto 22 mm diameter glass coverslips at a density of 1×10^5 cells/ml. After 1–2 days in culture cells were washed in Ca^{2+}-containing Krebs and loaded with 1 or 2 μM fura-2 acetoxy methyl ester for 30 min at room temperature. Cells were washed once in Ca^{2+}-containing Krebs and imaged after equilibration to 37°C for 3 min. Experiments were carried out at 37°C with continual perfusion of Ca^{2+}-containing Krebs buffer. Stimuli were applied to cells via a U-tube positoned to within 2 mm of the cell. Using this method the cells were challenged with the stimulus within 1 s of the onset of application. Fluorescent images were obtained by alternate excitation at 340 or 380 nm (40 ms each wavelength) using an image-processing system (Imagine, Synoptics Ltd, Cambridge) interfaced to a DEC microvax II microcomputer. The ratio image was obtained at video rate and filtered with a time constant of 200 ms. Determination of $[Ca^{2+}]_i$ was carried out using eqn 5 of Grynkiewicz et al. [20]. Contour maps were generated by Imagine from the ratio image and in all cases depict the distribution and rise in $[Ca^{2+}]_i$ elicited by the stimulus.

3. RESULTS AND DISCUSSION

The changes in $[Ca^{2+}]_i$ in individual chromaffin cells in response to various stimuli were examined using concentrations of the stimuli that were optimal for triggering secretion [8,13,21]. In experiments on fura-2-loaded chromaffin cells 10 μM nicotine and 55 mM K^+ released, over a 10 min period, 14.66 ± 0.32 and 10.20 ± 0.29%, respectively, of the total cellular catecholamine above basal, whereas 0.3 mM methacholine and 0.3 mM muscarine resulted in little, if any, secretion above basal (0.67 ± 0.09 and 0.36 ± 0.03%, respectively, of total cellular catecholamine) over the same period. These data confirm previous demonstrations that depolarizing stimuli [21], but not Ca^{2+}-mobilizing agonists [8,13], are potent secretagogues in these cells.

In all cells examined, initial stimulation with nicotine or high K^+ resulted in virtually immediate elevation in $[Ca^{2+}]_i$. Fig.1 shows the responses of two such cells to 10 μM nicotine (fig.1a) and 55 mM K^+ (fig.1b). In both cases, the mean $[Ca^{2+}]_i$ was elevated to at least 280 nM within 6 s. Although the maximum $[Ca^{2+}]_i$ in response to nicotine was maintained for longer (see below), both Ca^{2+} responses had returned to basal within 60 s. Similar responses to these drugs were observed in aequorin-loaded [21] and fura-2-loaded [12] cells. In addition to the magnitude of the responses, fig.1 also shows the distribution of the stimulus-induced rise in $[Ca^{2+}]_i$, in the form of 3-dimensional contour maps of the cell, at two time points during each response. The contour maps demonstrate that the Ca^{2+} response to both these stimuli originates in an area directly beneath the plasma membrane as at early time points after stimulation (4 s, nicotine; 2.5 s, high K^+) the rise in $[Ca^{2+}]_i$ is exclusively localized in the subplasmalemmal region of the cell (fig.1ai,bi). This would be consistent with a stimulus-dependent influx of extracellular Ca^{2+}. Further analysis revealed that at the peak of the response to depolarizing stimuli, one of two distributions of Ca_i^{2+} was typically observed (fig.1aii,bii): either the cell infilled and Ca_i^{2+} became homogeneously distributed throughout the cytoplasm, or the cell infilled with $[Ca^{2+}]_i$ then rising again in one pole of the cell. This secondary rise in $[Ca^{2+}]_i$ was initially observed in another study [12]. The cells in fig.1 were chosen to show an example of each distribution. Nicotine resulted in the secondary elevation of $[Ca^{2+}]_i$ at one pole of the cell whereas high K^+ resulted in a more uniform distribution of Ca_i^{2+}, but in other cells these responses were reversed. With the secondary rise in $[Ca^{2+}]_i$ a maximum of 484 nM was achieved in one pole of the cell and the overall signal was prolonged (fig.1a). Without this phase, a lower

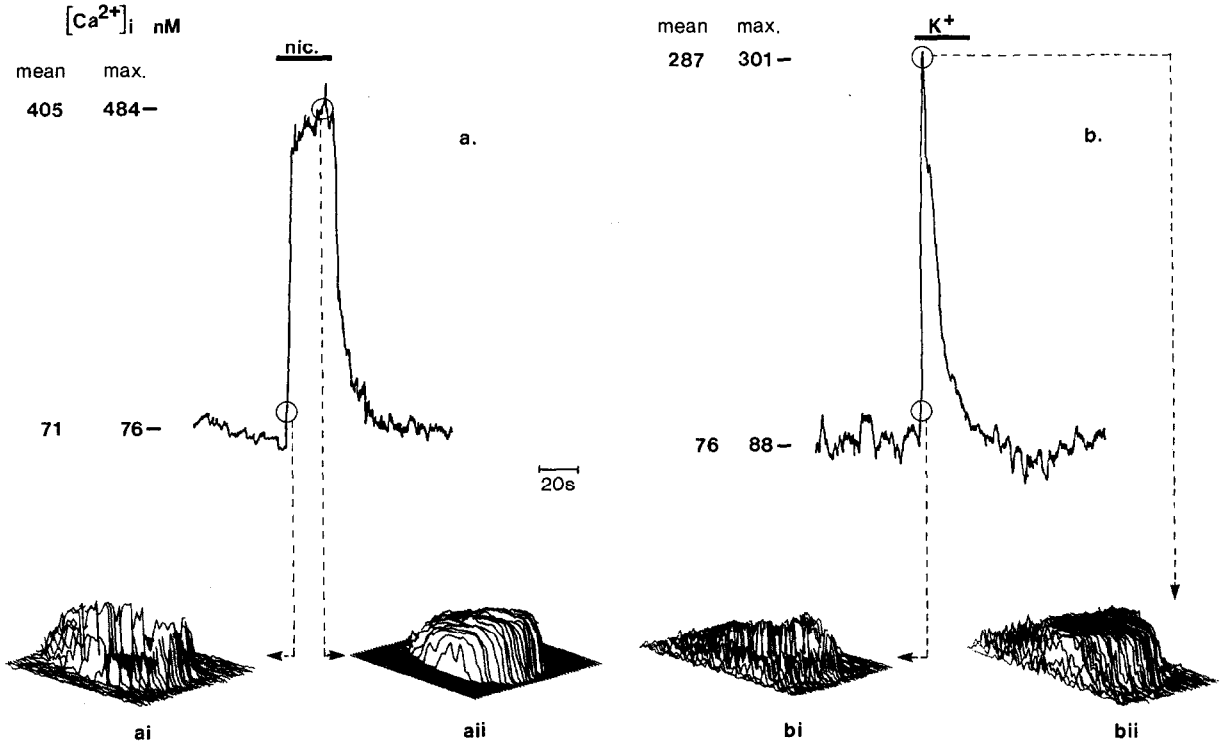

Fig.1. Time course and spatial localization of changes in $[Ca^{2+}]_i$ in single fura-2-loaded chromaffin cells in response to nicotine and high K^+. Time courses are photodiode recordings of video images and show responses characteristic of those due to 10 μM nicotine (a) and 55 mM K^+ (b). Contour maps of the cells show the localization of $[Ca^{2+}]_i$ at two times after stimulation and were derived by subtracting the values of $[Ca^{2+}]_i$ for the unstimulated condition (2 s prior to stimulation) from the value for the stimulated condition at the appropriate time. Mean $[Ca^{2+}]_i$ indicates the average $[Ca^{2+}]_i$ throughout the entire cell. Max $[Ca^{2+}]_i$ indicates the maximum $[Ca^{2+}]_i$ achieved at any one point within the cell.

maximum $[Ca^{2+}]_i$ of 301 nM was achieved throughout the cell and the signal was more transient (fig.1b). It is likely that the secondary rise in $[Ca^{2+}]_i$ results from Ca^{2+} being released from an internal store, since both nicotinic receptor activation [11] and high K^+ stimulation [11,22] resulted in the Ca^{2+}-dependent formation of inositol phosphates in chromaffin cell populations. This effect has been attributed to direct activation of phospholipase C by Ca_i^{2+} [23], but Ca^{2+}-induced Ca^{2+} release [29] cannot be ruled out.

Activation of the chromaffin cell muscarinic receptor also results in an elevation in $[Ca^{2+}]_i$ [7,8]. This result was also observed in the present study: 0.3 mM methacholine resulted in a 30 nM rise in $[Ca^{2+}]_i$ in a cell population (fig.2a), but no significant secretion above basal (see above). In view of the fact that full secretion is triggered by concentrations of nicotinic agonists which raise $[Ca^{2+}]_i$ to ~200 nM above basal (fig.1a and [8,21]), it has been assumed that the modest elevation of $[Ca^{2+}]_i$ elicited by muscarinic drugs is insufficient to trigger the exocytotic machinery [7,8,11]. This explanation now appears unlikely as the Ca^{2+} response of 10 single cells to a challenge with methacholine and then nicotine (fig.2b) clearly showed that in some cells methacholine was capable of eliciting a Ca^{2+} response with a peak value that approached that seen in response to nicotine (fig.2b, traces 2,4,11), whereas in other cells it elicited no Ca^{2+} response at all (fig.2b, traces 3,7,8). This result demonstrates that the muscarinic Ca^{2+} signal obtained from cell populations is not an accurate reflection of the changes in $[Ca^{2+}]_i$ that occur at the single-cell level. Evidence that a rise in $[Ca^{2+}]_i$ due to release of internal Ca^{2+} is not an effective trigger for secretion in these cells comes from the patch-clamp studies of Penner and Neher [3] who found

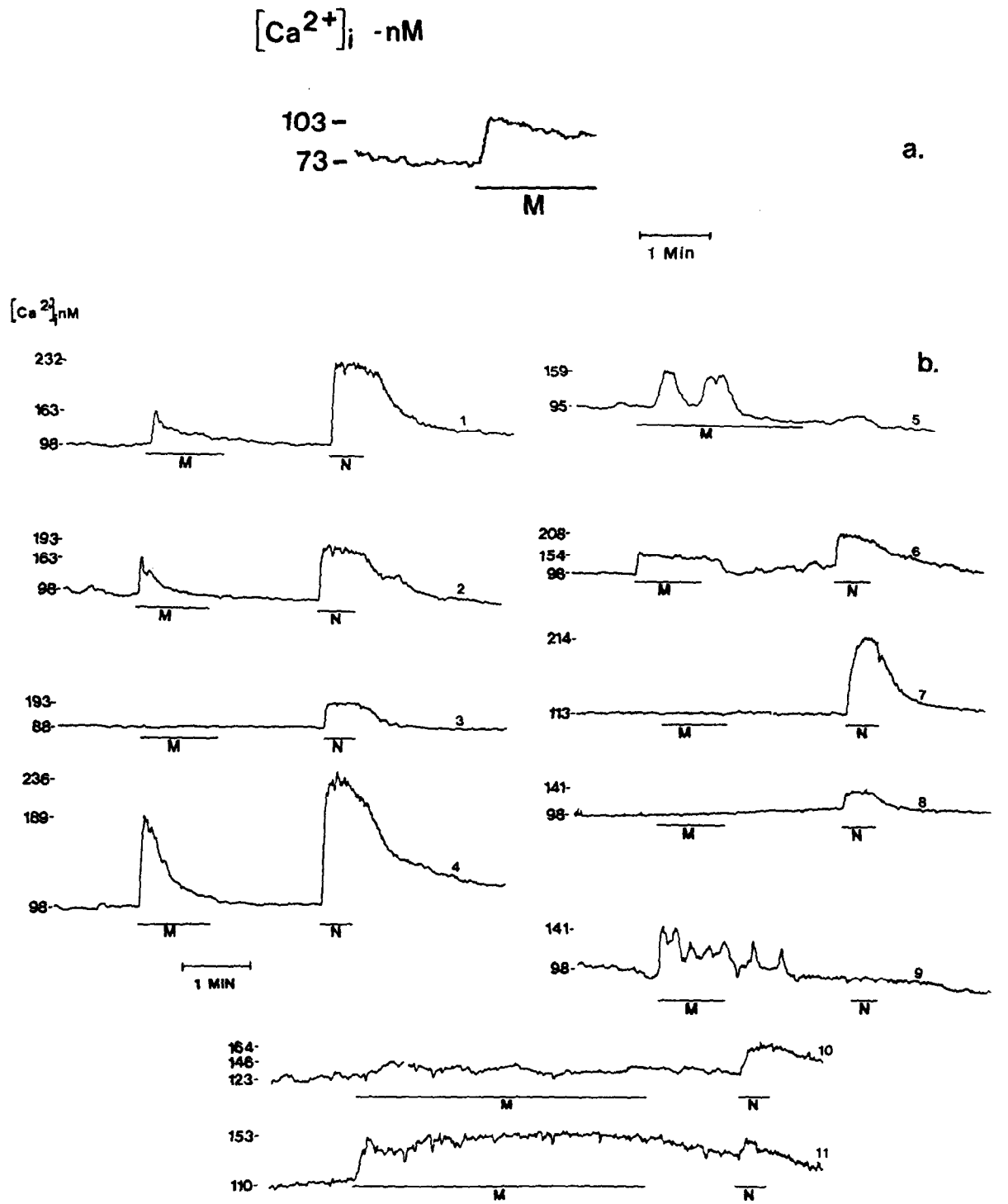

Fig.2. Time course of changes in $[Ca^{2+}]_i$ in chromaffin cells in response to methacholine. (a) Change in $[Ca^{2+}]_i$ in a population of fura-2-loaded cells in response to 0.3 mM methacholine (M), (b) photodiode recordings showing the change in $[Ca^{2+}]_i$ in 10 single cells in response to a challenge with 0.3 mM methacholine (M) and then 10 μM nicotine (N), and a single cell (trace 5) in response to 0.3 mM methacholine (M) only.

that direct activation of phospholipase C by intracellularly applied GTPγS resulted in large (>400 nM) transient rises in $[Ca^{2+}]_i$ but negligible secretion from chromaffin cells. The contour maps of cells responding to muscarinic agonists (fig.3a,b) clearly show that the distribution of Ca_i^{2+} was markedly different from that due to nicotine or high K^+ (fig.1a,b). Fig.3a shows a cell responding to 0.3 mM muscarine with a transient elevation in mean $[Ca^{2+}]_i$ of similar magnitude (390 nM) to that seen with nicotine (cf. fig.1a). In response to muscarine, the rise in $[Ca^{2+}]_i$ clearly originated from one pole of the cell (fig.3ai), and even at the peak of the response when $[Ca^{2+}]_i$ was maximal (500 nM) the majority of Ca_i^{2+} was still confined to the area from which it originated (fig.3aii). This distribution is strikingly different from the infilling effect seen in response to depolarizing stimuli and is likely to be a consequence of Ca^{2+} being released from a specifically localized internal store in the cell. In support of this notion, the presumptive Ca^{2+} store of non-muscle cells, the ER [24] (or calsiosome [25]), is often localized at one pole of the chromaffin cell [12]. Essentially the same polarized Ca_i^{2+} distribution was seen in a cell which responded strongly to the alternative muscarinic agonist methacholine (fig.3bi). The second contour map from this cell (fig.3bii) demonstrates a further point of interest, that in cells which respond to methacholine with more than one transient rise in $[Ca^{2+}]_i$ (e.g. see fig.2b) the distribution of Ca_i^{2+} at the peak of the second transient is the same as that at the peak of the first. This again indicates that Ca^{2+} released by muscarinic drugs originates only from specifically localized stores and, in addition, shows that the store is capable of rapidly refilling after initial depletion.

The reasons why the rise in $[Ca^{2+}]_i$ elicited by

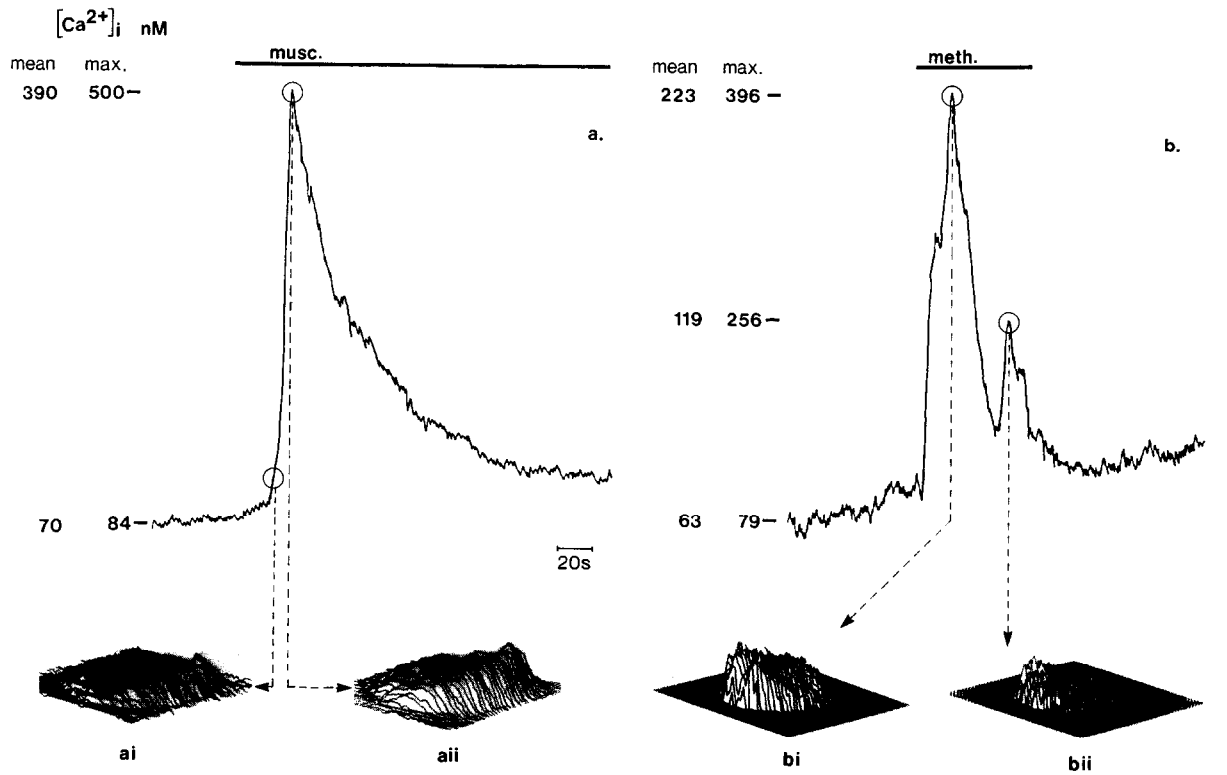

Fig.3. Time course and spatial localization of changes in $[Ca^{2+}]_i$ in single chromaffin cells in response to muscarinic receptor activation. Time courses are photodiode recordings of video images of cells which responded to 0.3 mM muscarine (a) and 0.3 mM methacholine (b). Contour maps of the cells show the localization of $[Ca^{2+}]_i$ at two times after stimulation and were derived by subtracting the values of $[Ca^{2+}]_i$ for the unstimulated condition (2 s prior to stimulation) from the value for the stimulated condition at the appropriate time. Mean $[Ca^{2+}]_i$ indicates the average $[Ca^{2+}]_i$ throughout the entire cell. Max $[Ca^{2+}]_i$ indicates the maximum $[Ca^{2+}]_i$ achieved at any one point within the cell.

muscarinic agonists remains, in the main, localized to one pole of the cell are unclear. Fig.3bii indicates one possibility, i.e. that internally released Ca^{2+} is rapidly resequestered. An alternative possibility is that Ca^{2+} is extruded from the cell as it is known that muscarinic, but not nicotinic, receptor activation results in rapid Ca^{2+} efflux from these cells [26]. One point which is clear however is that cells such as those in fig.3 are capable of responding to muscarinic agonists with strong rises in $[Ca^{2+}]_i$ without apparently stimulating the cell to secrete. It could therefore be that the restriction of the rise in $[Ca^{2+}]_i$ to one area (the ER?) of the cell is not conducive to either the triggering or maintenance of a subplasmalemmal event such as exocytosis in these cells.

A result of considerable significance therefore emerges from this study. This is that despite elevating $[Ca^{2+}]_i$ to similar levels in a cell, different classes of agonists can give rise to totally different spatial distributions of Ca_i^{2+}, a fact which could have important implications as regards the ability of the Ca^{2+} signal to trigger a given physiological response. In the case of exocytosis from the bovine chromaffin cell, it appears that the optimal signal for the triggering of secretion requires initial activation of the entire subplasmalemmal area in order to prime exocytotic sites, perhaps by disrupting the cortical cytoskeleton [27] and translocating protein kinase C to the plasma membrane [28], both of which are Ca^{2+}-requiring subplasmalemmal events that have been implicated in the secretory event. The elevation of $[Ca^{2+}]_i$ due to release from internal stores could regulate alternative cellular functions such as the stimulus-dependent increase in hormone biosynthesis.

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